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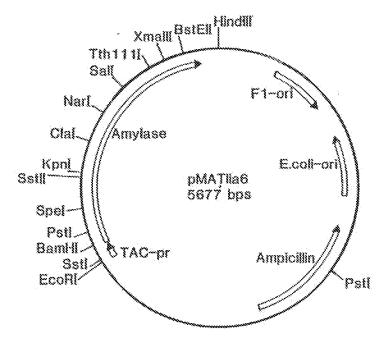
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(57) Abstract

Thermostable and acid stable a-amylases are provided as expression products of genetically engineered a-amylase genes isolated from microorganisms, preferably belonging to the class of Bacilli. Both chemical and enzymatic mutagenesis methods are e.g. the bisulphite method and enzymatic misincurporation on gapped heteroduplex DNA. The mutant a amylases have superior properties, e.g. improved thermostability over a broad pH range, for industrial application in starch processing and textile desizing.

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MUTANT MICROBIAL \(\alpha\)-ANYLASES WITH INCREASED THERMAL, ACID AND/OR ALKALINE STABILITY

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INTRODUCTION

Technical Field

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The present invention relates to the field of genetic engineering and provides new DNA molecules comprising DNA sequences coding for enzymes with a-amylase activity.

Specifically, mutant microbial a-amylases are disclosed

15 having improved characteristics for use in the degradation of starch, in the desizing of textile and in other industrial processes. The disclosed a-amylases show increased thermal, acid and alkaline stability which makes them ideally suited for performing their activity under process conditions which

20 could hitherto not be used.

Background of the invention

Starch consists of a mixture of amylose (15-30% w/w) and amylopectin (70-85% w/w). Amylose consists of linear chains of α -1,4-linked glucose units having a molecular weight (MW) from about 60,000 to about 800,000. Amylopectin is a branched polymer containing α -1,6 branch points every 24-30 glucose units, its MW may be as high as 100 million.

Sugars from starch, in the form of concentrated dextrose syrups, are currently produced by an enzyme catalyzed process involving: (1) liquefaction (or thinning) of solid starch with an a-amylase into dextrins having an average degree of polymerization of about 7-10, and (2) saccharification of the resulting liquefied starch (i.e. starch hydrolysate) with amyloglucosidase (also called glucoamylase or AG). The

resulting syrup has a high glucose content. Much of the glucose syrup which is commercially produced is subsequently enzymatically isomerized to a dextrose/fructose mixture known as isosyrup.

5 α-Amylase (EC 3.2.1.1) hydrolyzes starch, glycogen and related polysaccharides by cleaving internal α-1,4-glucosidic bonds at random. This enzyme has a number of important commercial applications in, for example the sugar, brewing, alcohol and textile industry. α-Amylases are isolated from a 10 wide variety of bacterial, fungal, plant and animal sources. The industrially most important α-amylases are those isolated from Bacilli.

In the first step of the starch degradation process, starch slurry is gelatinized by heating at relatively high 15 temperature (up to 110°C). The gelatinized starch is liquefied and dextrinized by a thermostable q-amylase in a continuous two stage process. The major process variables are starch concentration, a-amylase dose, temperature and pH. During the liquefaction-dextrinization reaction the process 20 variables must be maintained within narrow limits to achieve good conversion ratios, since serious filtration problems may arise otherwise. See, for example, L.E. Coker and K. Venkatasubramanian, in: Biotechnology, p. 165-171, Ed. P.N. Cheremisinoff, P.B. Quellette, Technicom Publ. Corp. 25 Lancaster Renn. 1985. One of the problems which frequently arises is the proper regulation of the temperature in the initial stage of the degradation process: overheating often causes denaturation of the o-amylase so that the final thinning is not sufficient. One way to avoid this is the use 30 of more thermostable a-amylases.

To that end it has been proposed to add calcium ions or an amphiphile (see e.g. EP-A-0189838), but this solution appeared to be unsatisfactory.

There is, therefore, still substantial interest to provide a-amylases with increased thermostability.

Relevant Literature

EP-A-057976 describes the isolation of a thermostable α-amylase coding gene from <u>B</u>. <u>stearothermophilus</u> the gene is cloned into a plasmid containing either a <u>Bacillus</u> or an <u>E</u>. <u>COLi</u> origin of replication. The so obtained chimeric plasmid is used for producing α-amylase. The α-amylase gene was isolated and used without any further modification.

EP-A-0134048 describes a method for increased commercial production inter alia of α-amylase, by cloning and expression of one or more α-amylase genes in industrial <u>Bacillus</u> strains.

EP-A-252666 describes a chimeric α-amylase with the general formula Q-R-L in which Q is a N-terminal polypeptide of 55 to 60 amino acid residues which is at least 75 percent homologous to the 37 N-terminal residues of the B. <u>Amyloliquefaciens</u> α-amylase, R is a given polypeptide and L is a C-terminal polypeptide of 390 to 400 amino acid residues which is at least 75 percent homologous to the 395 C-terminal residues of B. <u>licheniformis</u> α-amylase.

Gray et al. (J. Bacteriol., 1986, 166, 635) describe chimeric α -amylases formed of the NH₂-terminal portion of \underline{B} . Stearothermophilus α -amylase and the COOH-terminal portion of \underline{B} ·licheniformis α -amylase. Most of the hybrid enzyme

25 molecules were shown to be less stable than the parent wildtype enzymes. Furthermore none of the hybrid molecules was shown to possess improved stability properties.

None of the references cited above describes the use of single amino acid replacements to obtain novel α -amylases.

20 EP-A-0285123 discloses a method for complete mutagenesis of nucleic acid sequences. As an example mutagenesis of the B. <u>stearOthermophilus</u> a-amylase is described. Although there is a suggestion that this method can be used to obtain B. <u>stearOthermophilus</u> a-amylase mutants with improved stability 35 no examples are given.

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SUMMARY OF THE INVENTION

The present invention provides mutant q-amylases and ways of obtaining such mutants. Said mutant o-amylases are 5 characterized in that they differ in at least one amino acid from the wild-type enzyme. Furthermore, DNAs encoding these mutants, vectors containing these DNAs in expressionable form and host cells containing these vectors are provided.

In one aspect of the invention random mutagenesis on 10 cloned a-amylase genes is disclosed. The mutated genes are expressed in a suitable host organism using a suitable vector system.

In another aspect of the invention screening methods for mutant a-amylases are described and applied. Said methods yield more thermostable and more acid stable a-amylases. Furthermore, this method is used with a slight modification to obtain more alkaline stable q-amylases. The expression products of the clones so detected are isolated and purified.

In yet another aspect of the invention a-amylases are provided with increased thermostability, these mutant aamylases reduce filtration problems under application conditions of starch degradation.

In a further aspect of the invention α -amylases are provided with increased acid stability, these reduce the formation of unfavourable by-products, such as maltulose, at the same time they decrease the amount of acid to be added before the reaction with amyloglucosidase. The new a-amylases possess preferably both improved properties with respect to thermostability and acid stability or with respect to both 30 thermostability and alkaline stability.

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In another aspect of the invention the mutant proteins are shown to have a better performance under application conditions of starch liquefaction. The alkaline stability is especially useful for application in textile desizing.

These aspects will be further described in the detailed description and in the examples hereinafter.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Nucleotide sequence of pMa5-8

Stanssens <u>et al</u>., 1987, EMBO Laboratory Course

5 Martinsried, July 1987. For description of the different
elements see text.

Figure 2: Nucleotide sequence of plasmid ppRom Spo2 insert

Construction of this vector has been described in EP-A10 0224294. The \alpha-amylase amino acid sequence is depicted below
the triplets. Numbering starts from the first amino acid of
the mature protein (Kuhn et al., 1982, J. Bacteriol, 149,
372). The SPO2 promoter insert runs from position 61 to 344.

15 Figure 3: Nucleotide sequence of pMaTLia6

This vector was constructed from pMa5-8, the insert of pPROM SPO2 and a synthetic DNA fragment encoding the TAC promoter. The TAC promoter DNA fragment runs from position 3757 to position 3859. The a-amylase amino acid sequence is depicted below the triplets.

Figure 4 : Restriction map of pMaTLia6

The following unique restriction enzyme sites are available for gap construction in the a-amylase gene: BamHI, SpeI, SacII, KpmI, ClaI, NarI, SalI, ThtllII, XmaIII and EstEII. Sequencing primers for all possible gaps have been synthesized in order to enable easy determination of mutations. Plasmid pMcTLia6 is identical with pMaTLia6 except for the presence of an amber codon in the ampicillin gene (removes ScaI site) and the absence of an amber codon in the chloramphenicol gene (associated with the presence of a PvuII site).

Figure 5: Outline of Bacillus/E. coli shuttle vector pBMa/c

The (left) pMa/c section enables convenient mutagenesis
in E. coli. The (right) Bacillus subtilis cassette contains
the a-amylase gene (or any other Bacillus gene) plus a

minimal replicon for propagation in <u>B. subtilis</u>. After successful mutagenesis in <u>E. coli</u> the <u>B. subtilis</u> cassette can be circularized allowing the SPO2 promoter to move in front of the a-amylase gene upon transformation into <u>Bacillus</u>.

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Figure 6: Restriction map of pBMa/cl

This vector is a specific example of the mutagenesis expression vector outlined in Figure 5.

(1) and (2): multiple cloning sites. The target gene is
10 inserted in (2). By varying the sites at (1) and (2)
20 convenient restriction sites for gapped duplex creation can
20 be constructed;

FDT : transcription terminator

F1.ORI : origin of replication originating from

15 phage Fl

E. coli ORI: origin of replication from pBRJ22

BLA : ampicillin resistance gene

CAT : chloramphenicol resistance gene

BAC ORI : origin of replication of pUB110

20 KANAMYCIN : kanamycin (neomycin) resistance gene of

pUB110

SPO2 : promoter of phage SPO2

Figure 7: Restriction map of pBMa/c6Lia6

The <u>Bacillus licheniformis</u> α-amylase gene was engineered into pBMa/cl at multiple cloning site (2) of Figure 6. In this figure the SPO2 promoter is indicated by (2) and the <u>E. coli</u> ORI is represented by (4).

30 <u>Figure 8: Sequence of phoA signal sequence fragment in pMa/c</u> TPLia6

Depicted is the sequence from the $\underline{RCO}RI$ site upstream from the TAC-promoter up to the first amino acids of mature α -amylase. The phoA amino acid sequence is shown below the DNA sequence.

Figure 9: Michaelis-Menten plot for WT and 2D5 o-amylase

This plot shows the initial rate of enzyme activities
vs. substrate concentration for WT and 2D5 o-amylase. Assay
conditions are described in Example 8.

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Figure 10: Thermoinactivation of WT and D7 o-amylase

This plot shows the half life time of both WT and D7 α -amylase as a function of the ${\rm Ca}^{2+}$ concentration at pH 5.5 and 90.5°C.

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Figure 11: Thermoinactivation of WT and D7 a-amylase
As in Figure 10 except for the pH which is 7.0.

Figure 12: Thermoinactivation of WT and 2D5 a-amylase

This plot shows half life times of both WT and 2D5 α -amylase as a function of ${\rm Ca}^{2+}$ concentration at pH 7.0 and 95°C.

Figure 13: Thermoinactivation of WT and D7 α -amylase as a 20 function of pH

Figure 14: Thermoinactivation of WT and 2D5 α-amylase as a function of pH

25 Figure 15: DE vs final pH measured after liquefaction at 110°C

DETAILED DESCRIPTION OF THE INVENTION

30 By the term "exhibits improved properties" as used in connection with "mutant a-amylase" in the present description we mean a-amylases which have a higher enzymatic activity or a longer half-life time under the application conditions of starch liquefaction, textile desizing and other industrial processes.

With "improved thermostability" we mean that the mutant enzyme retains its activity at a higher process

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temperature, or that it performs longer at the same temperature than the wild-type enzyme from which it originates.

With "improved acid (or alkaline) stability" we mean that the mutant enzyme performs better at lower (or higher) pH values then the wild-type enzyme from which it was derived.

It is to be understood that the improved properties are caused by the replacement of one or more amino acids.

10

Chromosomal DNA may be isolated from an a-amylase containing microorganism. Preferably a microorganism is used belonging to the genus Bacillus, more preferably B. licheniformis, still more preferably B. licheniformis T5 is used (see EP-A-134048). The chromosomal DNA is digested with a suitable restriction enzyme and cloned into a vector. A number of possible ways of selection can be used e.g. hybridization, immunological detection and detection of enzymatic activity. The choice of the vector used for cloning the digested chromosomal DNA will depend on the selection method available. If hybridization is used no special precautions are needed. However, if detection is immunological or based on enzymatic activity the vector will have to contain the proper expression signals. The actual 25 detection of clones containing a-amylase was performed on starch containing agar plates. After growth and incubation with I, vapor halos are detected around positive clones. As a next step the sequence of the gene is determined. The derived amino acid sequence is used for comparison with other known a-amylase sequences to give a first impression of important amino acids (e.g. active-site, Ca2 binding, possible S-S bridges). A better indication is obtained when the 3Dstructure is determined. Since this is very laborious oftentimes another approach is used. In the absence of a 3D-35 structure prediction programs for determining the secondary structural elements (e.g. a-helix, 8-sheet) are successfully used eventually the tertiary structural elements e.g. 8-

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barrel are determined. For a review see Janin, J. and Wodack, S.J., Prog. Biophys. molec. Biol. 1983, 42, 21-78.

Valuable amino acid replacements can be envisioned.

The stability of a protein structure is determined by the net

difference in free energy between the folded and unfolded conformations of the protein. Since the proline residue is restricted to fewer conformations than the other amino acids the configurational entropy of unfolding a protein is decreased (and stability thereby increased) when an amino

acid is replaced with proline. Another useful substitution is the glycine to alanine replacement. Residues such as threonine, valine and isoleucine with branched 8-carbons restrict the backbone conformation more than non-branched residues.

Since a part of the thermostability of certain proteins is due to salt bridges it may be advantageous to introduce lysine and arginine residues (Tomozic S.J. and Klibanov A.M., J. Biol. Chem., 1988, 263 3092-3096). Moreover replacement of lysine by arginine residues may improve the stability of salt bridges since arginine is able to form an additional H-bond. For a review see Wigby, D.B. et al. Biochem. Biophys. Res. Comm. 1987, 149, 927-929. Deamidation of asparagine and glutamine is mentioned to cause a serious disruption of the enzyme structure, replacement with non-amide residues may avoid this disruption. Amino acid replacements are best made by mutagenesis at the DNA level.

In principle mutagenesis experiments can be performed immediately on isolated clones. However, the insert is preferably cloned in a mutagenesis/expression vector. Random mutagenesis is possible and so is site-directed mutagenesis. In view of the huge amount of mutated clones of the former method, and since no 3D-structure of a-amylase is known to make possible an educated guess for site-directed mutagenesis we decided to perform "random" mutagenesis in specific regions.

The following is a possible approach for practising the present invention.

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First the gene is modified by the introduction of "silent" restriction sites. Introduction of non-silent restriction sites is also possible. This makes possible the deletion of specific regions of the gene. Secondly the gene is cloned in a phasmid. This combination of a phage and a plasmid makes easy the production of single stranded DNA. Other ways of obtaining single stranded DNA are also possible. By hybridizing melted double-stranded vector (plus insert) DNA with a vector/insert combination containing a gap in the insert, gapped heteroduplex DNA was obtained (for a detailed description see Morinaga, Y et al. 1984, Biotechnology, 2, 636).

The gap is used for chemical or enzymatic mutagenesis. Preferably we used the bisulphite method (Folk and 15 Hofstetter, Cell, 1983, 33, 585) and an enzymatical misincorporation method are used (modified version of Lehtovaara <u>et al</u>., Prot. Eng., 1988, <u>2</u>, 63). These methods can be applied in such a way that every single nucleotide in the gap is replaced by all three other nucleotides (saturation mutagenesis). The latter method can be applied in 20 several ways. In one of them a synthetic primer is hybridized to the gap. Subsequently an extension reaction is performed in which the deoxynucleotide complementary to the first deoxynucleotide 3' from the primer is missing. In principle 25 all three of the other deoxynucleotides can thus be incorporated. This can be achieved either by using a mix of three deoxynucleotides or by using three separate reactions each containing only one deoxynuclectide. Another way of applying the method yields random clones. Here, four separate 30 reactions are set up each of them containing one limiting deoxynucleotide. This gives second strands that stop before every single nucleotide. The subsequent steps can be performed as described above. Both the bisulphite and the enzymatic mutagenesis method were employed to obtain mutants.

For testing the enzymatic properties it may be convenient to express the cloned genes in the same host as that used during mutagenesis experiments. In principle this

can be any host cell provided that suitable mutagenesis/expression vector systems for these cells are available. For the most part E. coli is very convenient to work with, for example E. coli WK6. After growth of the 5 colonies in microtiterplates samples from the wells of these plates are spotted on agar plates supplemented with starch and buffered at different pH values. Positive clones can be detected by halo formation. Screening with appropriate buffers can be used to select for thermostability, acid stability, alkaline stability, saline stability or any other stability that can be screened for.

Suitable host strains for production of mutant amylases include transformable microorganisms in which the expression of a-amylase can be achieved. Specifically host strains of the same species or genus from which the a-amylase is derived, are suited, such as a <u>Bacillus</u> strain. Preferably an a-amylase negative <u>Bacillus</u> strain is used more preferably an a-amylase and protease negative <u>Bacillus</u> strain.

For example B. <u>licheniformis</u> T9 has been used to produce high amounts of mutant a-amylases.

Preferably, the a-amylases being produced are secreted into the culture medium (during fermentation), which facilitates their recovery. Any suitable signal sequence can be used to achieve secretion.

The expressed a-amylase is secreted from the cells and can be subsequently purified by any suitable method.

Gelfiltration and Mono Q chromatography are examples of such methods. The isolated a-amylase was tested for thermoinactivation at different Ca concentrations (0.5 - 15 mM)

30 and over a wide pH range (5.5 - 8.0). Tests were also performed under application conditions. Specifically mutant a-amylase was tested under conditions of starch liquefaction at pH 5.5 and 5.25. Furthermore, applications for textile desizing have been tested.

The properties of some of the mutants that are screened will be better suited under the desired performance conditions.

The present invention discloses a-amylases with increased thermostability, improved acid stability and improved alkaline stability. Generally the number of amino acid replacements is not important as long as the activity of the mutated protein is the same or better than that of ther wild-type enzyme. Mutant a-amylases differ in at least one amino acid from the wild-type enzyme, preferably the mutants differ in from 1 to 10 amino acids. Specific mutants with improved properties include mutant a-amylases containing one or more amino acid replacements at the following positions 111, 133 and 149 (numbering is in accordance with the B. licheniformis a-amylase). Among the preferable amino and replacements are Ala-111-Thr, His-133-Tyr amd Thr-149-Ile.

Such mutant enzymes show an improved performance at pH values below 6.5 and/or above 7.5. The performance is also increased at high temperatures leading to an increased half-life-time at for example temperatures of up to 110°C.

Many of the available α-amylase products are obtained from bacterial sources, in particular Bacilli, e.g. B.

20 <u>subtilis</u>, B. <u>licheniformis</u>, B. <u>stearothermophilus</u>,
B. <u>coagulans</u> and B. <u>amyloliquefaciens</u>. These enzymes show a high degree of homology and similarity (Yuuki <u>et al</u>., J. Biochem., 1985, <u>98</u>, 1147; Nakajima <u>et al</u>., Appl. Microbiol. Biotechnol., 1986, <u>23</u>, 355). Therefore knowledge of

25 favourable mutations obtained from one of these α-amylases can be used to improve other amylases. The present invention

provides an approach for obtaining such knowledge.

Following is a description of the experimental methods used and examples to illustrate the invention. The examples 30 are only for illustrative purpose and are therefore in no way intended to limit the scope of the invention.

EXPERIMENTAL

35 Materials and Methods

1. General cloning techniques

Cloning techniques were used as described in the handbooks of T. Maniatis et al., 1982, Molecular Cloning, Cold Spring Harbor Laboratory; F.M. Ausubel et al., 1987,

5 Current Protocols in Molecular Biology, John Wiley & Sons Inc., New York; B. Perbal, 1988, A practical Guide to Molecular Cloning, 2nd edition, John Wiley & Sons Inc., New York. These handbooks describe in detail the protocols for construction and propagation of recombinant DNA molecules,

10 the procedures for making gene libraries, the procedures for sequencing and mutating DNA and the protocols for the enzymatic handling of DNA molecules.

15 2. <u>Chemical mutagenesis</u>

Cloned DNA may be treated in vitro with chemicals in order to introduce mutations in the DNA. If these mutations are directed to amino acid encoding triplet codons a mutated protein can be produced by the mutated cloned DNA. A method for chemical mutagenesis with the aid of sodium bisulfite is described by Shortle and Botstein (Methods Enzymol., 1983, 100, 457). A preferable method is described by Folk and Hofstetter (Cell, 1983, 33, 585). Other methods for mutagenesis are described by Smith, Ann. Rev. Genet., 1985, 19, 423. A particularly useful protocol is described by Ausubel et al., ibid.

30 3. <u>Mutagenesis on gapped-duplex DNA</u>

A method based on the gapped-duplex approach (Kramer <u>Et al.</u>, 1984, Nucl. Acids Res. 12, 9441) and a phasmid (plasmid/phage hybrid) was used. Essentially the method rests on a gapped duplex DNA intermediate consisting of a gapped strand (-strand) containing a wild-type antibiotic resistance marker and a template strand (+ strand) carrying an amber mutation in the gene conferring resistance to the antibiotic.

After annealing, the mutagenic oligonucleotide becomes incorporated in the gapped strand during in vitro gap-filling and sealing reaction. The resultant molecules are used to transform a mismatch repair deficient (Mut S) host in which the linkage between the intended mutation and the antibiotic resistance marker is preserved. The mixed phasmid population, isolated from this strain, is then allowed to segregate in a suppressor negative host strain. Transformants are plated on antibiotic containing medium, thus imposing a selection for progeny derived from the gapped strand.

The twin vector system pMa/c5-8, which was described by P. Stanssens et al. (Nucl. Acids Res., 1989, 17, 4441) is composed of the following elements:

15 pos 11-105 : bacteriophage fd, terminator

pos 121-215 : bacteriophage fd, terminator

pos 221-307 : plasmid pBR322 (pos 2069-2153)

pos 313-768 : bacteriophage fl, origin of replication

(pos 5482-5943)

20 pos 772-2571 : plasmid pBR322, origin of replication

and 8-lactamase gene

pos 2572-2685: transposon Tn903

pos 2519-2772: tryptophan terminator (double)

pos 2773-3729: transposon Tn9, chloramphenicol acetyl

25 transferase gene

pos 3730-3803: multiple cloning site

The sequence is depicted in Figure 1.

In the pMa type vector nucleotide 3409 is changed from G to A, while in the pMc type vector nucleotide 2238 is changed from G to C, creating amber stopcodons in the acetyl transferase gene and S-lactamase gene, respectively, rendering said genes inactive.

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All sequences referred to were obtained from Genbank (TM) (release 54), National Nucleic Acid Sequence Data Bank,

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NIH USA. Plasmid pMc5-8 has been deposited under DSM 4566. To perform mutagenesis the target DNA fragment is cloned into the multiple cloning site of pMa5-8. Subsequently a gapped duplex between pMa5-8 containing the target DNA and pMc5-8 is 5 constructed.

The single strand gap, consisting of the target DNA, can be subjected to mutagenesis with a mutagenic oligonucleotide, with long synthetic oligonucleotides, with a low level of misincorporated nucleotides, with chemicals or with enzymatic misincorporation of nucleotides also random mutagenesis PCR can be applied. For a detailed description see Ausubel et al., ibid. or Perbal, ibid. As an alternative to in vitro mutagenesis one can use in vivo mutagenesis either with the aid of UV-light or chemicals or by the application of an E. coli mutator strain (Fowler et al., J. Bacteriol., 1986, 167, 130).

Mutagenic nucleotides can be synthesised using apparatus obtainable from Applied Bio Systems.

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Random mutanenesis by enzymatic misincorporation of nucleotides

A pMa/pMc gapped duplex can be subjected to primer 25 extension and misincorporation mutagenesis as originally described by Shortle et al. (Proc. Natl. Acad. Sci. USA, 1982, <u>79</u>, 1588) by B.C. Cunningham and J.A. Wells (Prot. Eng., 1987, 1, 319) a modification of this procedure is described by Lehtovaara et al., (Prot. Eng., 1988, 2, 63).

This method is based on controlled use of polymerases. Four populations of DNA molecules are first generated by primer elongation of a gapped duplex of pMa/pMc so that they terminate randomly, in the gap, but always just before a known type of base (before A, C, G or T, respectively). Each 35 of four populations is then mutagenized in a separate misincorporation reaction where the correct base can now be omitted. In this way all types of base substitution mutations WO 91/08353 PCT/EP90/01042

can be generated at every position of the gap. The use of sequenase (TM) (U.S. Biochemical Corporation) was preferred to the use of Klenow polymerase. Moreover MoMuLV reverse transcriptase was used instead of A.M.V. reverse 5 transcriptase, which was used by Lehtovaara et al. (ibid).

To ensure single site substitutions we have introduced the following modification to the protocol described by Lehtovaara et al., ibid. In the reverse transcriptase buffer not three but only one misincorporating nuclectide is 10 present. For instance the A-specific limited base elongation mixture is incubated in three separate reactions with 250 μM dCTP, 250 µM dGTP and 250 µM dTTP, respectively. For a complete set of 4 base specific limited elongation mixtures a total set of 12 separate misincorporation reactions is 15 carried out. After 1.5 hour incubation at 42°C a chase of all four decxynuclectides in a concentration of 0.5 mM is added and the reactions are further incubated for at least 20 minutes at 37°C. Samples are then further processed according to Lehtovaara et al. (ibid.), with the modification that no 20 counterselection to an uracil-containing DNA strand but a counterselection based on the pMa/c vector was applied.

Production of mutant a-amylases

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Transformants of Ε. <u>coli</u> strain WK6 (Zell, R. and Fritz, H.J., EMBO J., 1987, 6, 1809), containing an expression vector, harboring any one of the α-amylase constructs, were inoculated in TB medium (10 ml) at 30°C. TB medium consisted of 0.017M KH₂PO₄, 0.072M K₂HPO₄, 12 g/l Bactotryptone, 24 g/l Bacto yeast extract, 0.4% glycerol and an antibiotic (ampicillin with pMa or chloramphenical with pMc constructs). Samples of the culture were used to inoculate 250 ml TB in 2 liter flasks. At an OD₆₀₀ of 10 - 12, 0.1 mM IPTG (isopropyl-B-d-thiogalactopyranoside) was added and incubation continued for another 12 - 16 hours.

6. Purification of mutant α-amylases

- The cells were harvested by centrifugation and resuspended in buffer containing 20% sucrose at 0°C. After a second centrifugation the cells were resuspended in cold water. Cell debris was removed by a third centrifugation and the supernatant was brought to pH 8.0 with 20mM TRIS buffer.
- CaCl $_2$ was added to a final concentration of 50mM. The material was heat-treated for 15 min. at 70°C and the insoluble material removed by centrifugation. The supernatant was filtered through 0.22 μ Millipore filter and concentrated to 1/10th of the starting volume.
- Further purification was achieved using gelfiltration (on TSK HW-55- Merck) and Mono Q chromatography. Before chromatography on Mono S the pH, of the enzymatic activity containing fractions, was adjusted to 4.8 using sodium acetate. α-amylase was eluted with 250mM NaCl. To avoid
- 20 inactivation the pH was immediately adjusted to 8.0.

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Examples

Example 1

Molecular cloning of Bacillus licheniformis a-amylase gene

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Chromosomal DNA isolated from Bacillus licheniformis T5 (EP-A-134048; CBS 470.83) was digested with restriction enzyme <u>EcoRI</u> and ligated into the <u>Eco</u>RI site of pUB110 (Gryczan, T.J., et al., J. Bacteriol, 1978, 134, p 318). The 10 ligation mixture was transformed into <u>Bacillus subtilis</u> 1A40 (Bacillus Genetic Stock Center). Neomycine resistant colonies were tested for a-amylase production on HI agar plates (DIFCO) supplemented with 0.4 g/l starch (Zulkowsky starch, Merck). After growth and incubation with I, vapor, a positive 15 colony producing a large clearing halo was selected for further characterization. The plasmid isolated from this positive colony was shown to contain a 3.4 kb EcoRI-EcoRI fragment originating from <u>Bacillus licheniformis</u> T5. This plasmid was named pGB33 (EP-A-134048; CBS 466.83). The α -20 amylase encoding insert was ligated to a synthetic Shine-Dalgarno sequence and the bacteriophage SPO2 promoter resulting in plasmid pProm SPO, (see EP-A-0224294; CBS 696.85). The nucleotide sequence of the insert of pProm SPO, as determined by the method of Sanger (Proc. Natl. Acad. Sci. 25 U.S.A., 1977, 74, 6463) is shown in Figure 2. The sequence shows a single large open reading frame encoding an ${\mathfrak a}$ amylase, which is virtually identical to the q-amylase sequence of <u>Bacillus licheniformis</u> as determined by Yuuki et al. (ibid). The first 29 amino acids are a signal sequence 30 Which is cleaved off during secretion of the a-amylase. Numbering of amino acids throughout this application refers to the numbering according to the mature protein.

The Yuuki sequence differs at the following positions: at position 134 an Arg is present instead of Leu; at position 35 310 a Ser is present instead of Gly; at position 320 an Ala is present instead of Ser.

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m 39 m

Example 2

Construction of mutagenesis/expression vectors pMaTLia6

33 Plasmid pPROM SPO, was digested with EcoRI and EclI and the 1.8 kb <u>Eco</u>RI-<u>Bol</u>I insert was purified and cloned into EcoRI-BamHI digested pMa5-8. This pMa5-8 vector was beforehand provided with a modified multiple cloning site. The BamHI-HindIII fragment running from position 3767 to position 3786 in Figure 1 was exchanged for a synthetic DNA sequence as it reads from position 5647 to 5660 in Figure 3. This was carried out to render some restriction sites within the camylase gene unique. The resulting o-amylase containing pMa5-8 derivative was digested with EcoRI and BamHI and ligated to a synthetic DNA fragment carrying a copy of the TAC promoter (De Boer et al., Proc. Natl. Acad. Sci. U.S.A., 1983, 80, 21). The sequence of this synthetic DNA fragment is depicted together with the final a-amylase mutagenesis/expression vector pMaTLia6 in Figure 3 from position 3757 to position 3859. This final a-amylase mutagenesis/expression vector was completed by the introduction of several silent restriction sites which are intended to produce gaps in the c-amylase gene during mutagenesis experiments (Figure 4). For this purpose the following mutations have been made using sitedirected oligonucleotide mutagenesis:

> - a SpeI site has been introduced by a silent mutation:

T49T and S50S

ACG --> ACT AGC --> AGT

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- a NarI site has been introduced by the silent mutation:

A269A

GCG --> GCC

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- A BstE II site has been introduced just downstream from the TAG stop codon

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TAGAAGAGC --> TAGGTGACC

This a-amylase mutagenesis vector pMaTLia6 is suited for mutagenesis with the gapped duplex method. Double stranded pMaTLia6 DNA prepared by digestion of suitable restriction enzymes has been annealed to single stranded pMcTLia6 DNA.

The resulting single stranded gaps have been subjected to site-directed mutagenesis, to chemical mutagenesis and to random enzymatic mutagenesis as described in the experimental 10 section.

The availability of the TAC promoter in front of the α -amylase gene enables the inducible expression of α -amylase in E. <u>CQLi</u> by addition of IPTG.

Plasmid pMaTLia6 in E. <u>coli</u> WK6 was deposited as CBS 15 255.89 on June 2nd, 1989.

Example 3

20 <u>Construction of a Bacillus/E. coli shuttle vector</u> <u>for mutagenesis and expression</u>

This vector enables mutagenesis of an inserted gene in E. COLI and immediate expression in Bacillus. The strategy chosen for the construction of the vector was to combine a pUB110 derivative (Gryczan, ibid.) with the pMa/c twin vector system in such a way that:

- The B. <u>subtilis</u> cassette can be removed by a single restriction/religation experiment.
- 2. Different a-amylase genes and different promoters can be easily cloned in this vector.
- 3. After recircularisation the cloned gene will be under control of a suitable Bacillus promoter.
- 4. During mutagenesis in <u>E. coli</u> the Bacillus

 promoter and the structural a-amylase gene are
 physically separated preventing a possible lethal
 accumulation of a-amylase in <u>E. coli</u>.

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A schematic drawing of the shuttle vector is shown in Figure 5. The structure of the final version of the vector pBMa/cl is depicted in Figure 6. Vector pBMal has been deposited under number CBS 252.89, on June 2nd, 1989. The vector has been constructed as follows:

- The <u>EcoRI-SnaBI</u> fragment of pUB110 carrying the REFgene and the Neo[®] gene was purified and cloned into <u>EcoRI-Sma</u>I digested pUC8.
- The <u>Rco</u>RI-<u>Hin</u>dIII fragment of this pUCS derivative was cloned into <u>Eco</u>RI-<u>Hin</u>dIII digested pMa5-8 resulting in plasmid pMa5-80.
- The <u>RamHI-Xbal</u> polylinker fragment was substituted by a synthetic fragment of DNA encoding the SPO₂ promoter of bacteriophage SPO₂ (Williams <u>et al.</u>, J. Bacteriol., 1981, <u>146</u>, 1162) plus restriction recognition sites for <u>Sac</u>II, <u>Apal</u>, <u>XhoI</u>, <u>Sac</u>I, <u>BglI</u>, <u>MluI</u> and <u>Xba</u>I.
- The unique <u>Eco</u>RI site of pMa5-80 was used to insert a polylinker fragment constituting the following recognition sites: <u>EcoRI</u>, <u>SmaI</u>, <u>EcoRI</u>, <u>SphI</u>, <u>KpnI</u>, XbaI and <u>HindIII</u>

For specific purposes derivatives pBMa/c2 and pBMa/c6 have been developed out of pBMa/c1.

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- In pBMa/c2 the EcoRI-HindIII polylinker of pBMa/cl has been replaced by the corresponding polylinker of pUC19.
- In pBMa/c6 in addition the <u>Sac</u>II site in the right polylinker of pBMa/c1 has been removed by a Klenow reaction.

Site directed mutagenesis on the B. <u>licheniformis</u> α amylase gene was performed after construction of pBMa/c6

35 Lia6. This vector was constructed by ligating the <u>Bam</u>HI<u>Hin</u>dIII fragment isolated from pMaTLia6 into the above
mentioned pBMa/c6 which was cleaved by <u>Bam</u>HI and <u>Hin</u>dIII. The

resulting plasmid (Figure 7) can be used to construct gapped duplexes for mutagenesis in E. coli.

The resulting mutants have been expressed in <u>Bacillus</u>
<u>Subtilis</u> 1A40 (BGSC 1A40) after restriction with <u>Sac</u>I,

5 religation and transformation according to Chang and Cohen
(Mol. Gen. Genet., 1979, <u>168</u>, 111).

Example 4

10 Expression in E. coli of correctly matured
Bacillus licheniformis a-amylase

Characterization of the α -amylase produced by pMaTLia 6 (Example 2) showed that a portion of the α -amylase was 15 incorrectly processed during secretion. NH₂-terminal sequencing revealed an extra Alanine residue for α -amylase produced in E. coli WK 6.

Although we have no indication that this will give different properties to the amylase we have replaced the a
20 amylase signal sequence by the alkaline phosphatase PhoA signal sequence. To this end a mutagenesis experiment was carried out so as to introduce a FspI restriction site in pMaTLia 6 at the junction of the signal peptide and the mature a-amylase. After FspI and BamHI digestion a synthetic DNA fragment encoding the phoA signal sequence (Michaelis et al. J. Bacteriol., 1983, 154, 366) was inserted. The sequence of this construction is shown in Figure 8. a-Amylase produced by pMa/cTPLia6 was shown to posses the correct NH2-terminal sequence.

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Example 5

Screening for stable q-amylase

A. Screening for acid-stable q-amylase mutants

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 $\alpha\textsc{-Amylase}$ mutants, that perform better or worse at low pH than the wild-type $\alpha\textsc{-amylase}$, can be selected by

comparison of halo's on starch plates buffered at different pH values after staining the starch with an iodine-solution.

Method:

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1. Growth

Possible mutants are grown in microtiterplates. The growth medium is 250 μl Brain Heart Infusion broth (DIFCO). The following additions are made:

10 chloramphenicol 50 μ g/ml

I.P.T.G. (SIGMA) 0.2 mM

CaCl, 2 mM

Colonies are picked from agar plates with sterile toothpicks and inoculated in separate wells (96) of a microtiterplate.

In each plate 4 wild-type colonies are included as a control. These microtiterplates are placed at 37°C for 40 hours without shaking.

2. Plate test

- After this time period, in which the α-amylase is produced, 5 μl samples are taken from each well and spotted on 2 different types of agar plates (144 x 140 mm). The first type is a rich Heart-Infusion agar plate (DIFCO) + 0.4% starch (Zulkowsky starch-Merck) + chloramphenicol 50 μg/ml.
- 25 After incubation at 37°C for 16 hours this plate serves as a storage for mutants.

The second type of plate is the actual screening plate, it contains: Bacto agar (DIFCO) 1.5%

Zulkowsky starch 0.2%

30 Agar and starch are dissolved in synthetic tap water (STW). This is: demineralised water +

CaCl, 2 mM

MgCl, 1 mM

NaHCO₃ 2.5 mM

35 BSA 10 μg/ml

The screening plates are buffered by a 100-fold dilution of a 5 M stock potassium acetate buffer solution in this medium. pH values of the stock solutions are 4.80; 5.0 and 5.2 at room temperature. Final pH values in the agar plate when measured are somewhat lower than those of the stock solutions. From each well 5 µl of culture is spotted on 3 screening plates with different pH values.

The pH-range is chosen in such a way that there is little or no activity left for the wild-type α -amylase on the 10 plate with the lowest pH-value.

3. Colouring

The screening plates are incubated for 2 hours at 55°C. After this period an $\rm I_2$ solution is poured over the 15 plates. 10 x $\rm I_2$ solution contains 30 g $\rm I_2$ and 70 g KI per liter.

The amount of clearance of the spots is correlated with the residual a-amylase activity at that pH value. Those mutants that perform better than the wild-type controls are 20 selected for a second round of screening. Wild-type halo's are very reproducible in this experiment.

4. Second screening

Positive mutants are picked from the rich plate and
purified on fresh HI plates + chloramphenicol. 4 single
colonies are picked from each mutant and they are tested
again in a similar way as in the first screening. In addition
serial dilutions of these cultures are made with STW and
these dilutions are spotted on neutral pH screening plates
(pH = 7.0). Comparison with wild-type cultures enables one to
decide if the better performance at low pH is due to an
overall better a-amylase production or to intrinsically more
stable a-amylase.

The mutants that "survive" the second screening are

35 characterized by determining the nucleotide sequence of that

part of the gene that was subjected to mutagenesis.

B. Screening for alkali stable o-amylase

Screening for alkali stable α -amylases is performed in a manner similar to the one used for acid stable α -amylase. 5 After growth in microtiter plates 5 μ l samples are taken from each well and spotted onto a storage plate and onto the actual screening plate. The latter is composed of:

Bacto Agar (DIFCO) 1.5%
10 Zulkowsky starch 0.2%

and completed with demineralized water plus

CaCl2 2 mM
15 MgCl2 1 mM
NaHCO3 2.5 mM
BSA 10 µg/ml

The screening plates are buffered with 50 mM

20 carbonate/bicarbonate buffer, pH values are 9.0, 9.5 and
10.0. The pH range is chosen in such a way that there is
little or no activity of the wild-type a-amylase at the
highest pH value. After 2 hours incubation at 55°C an I2
solution is poored over the plates. Those mutants that give a

25 better halo than the wild-type enzyme are selected for a
second round of screening. This second round of screening is
performed in a similar fashion as the screening for the acid
stability.

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C. Screening for thermostable o-amylase mutants

α-Amylase mutants that perform better or worse at high temperature than the wild-type α-amylase, can also be
35 selected by comparison of halo's on starch plates caused by the residual amylase activity in the culture broths after heating.

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Method:

 Mutants are grown in the same way as for the pHscreening.

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- 2. The mutants are replicated on HI agar plates as for the pH-screening.
- 3. The separate wells of the microtiterplates were closed with disposable caps (Flow laboratories) to prevent evaporation of the culture broths during the heating step.
- 4. Microtiterplates were heated in a waterbath for 1 hour 15 at 95°C. After heating the microtiterplates were placed in a centrifuge for collecting the total sample on the bottom of the microtiterplate.
- Screening for thermostable mutants was done as
 follows:

From each well 5 μ l of culture was spotted on neutral screeningplates (See pH-screening). These plates were incubated for 1 hour at 55°C.

After staining the starch with the iodine solution

mutants and controls can be screened for residual o
amylase activity by comparing clearance of the spots
(halo's).

In case the residual activity of the controls is too high, serial dilutions must be made and spotted on the screening plate to be able to discriminate for mutants that are more thermostable than the wild-type enzyme.

 Possible interesting mutants are tested further as was done in the pH-screening method.

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A combination of screening type A or B with type C can be applied if a combination of properties is desired. For

instance after the first round of screening for alkali stable a-amylase, a second round of screening for thermostability can be performed. Those mutants that score positive in both tests may be selected as candidates exhibiting a combination of desired properties.

Example 6

Bisulphite mutagenesis of pMaTLia6

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Single stranded DNA of pMaTLia6 was amnealed with SacII-ClaI digested pMcTLia6 in order to obtain a heteroduplex with a gap running from position 4315 to 4569 (Figure 3). This heteroduplex was subjected to bisulphite mutagenesis (see experimental).

After transformation into E. <u>Coli</u> WK6 mut S (Zell, R. and Fritz H.J., ibid) and selection on chloramphenical containing agar plates (50 µg/ml) plasmid pools were isolated and transformed into E. <u>Coli</u> WK6. E. <u>Coli</u> WK6 Mut S was deposited as CBS 472.88, E. <u>Coli</u> WK6 was deposited as CBS 473.88. Resulting transformants were grown in BHI medium (DIFCO) containing 2.0 mM CaCl₂, 50 µg/ml chloramphenical and 0.20 mM IPTG (SIGMA) during 40 hours at 37°C in microtiter wells without shaking. Screening for pH stable mutants was carried out as described in Example 5.

About 300 Cm⁸ transformants were screened. The mutation frequency as determined by DNA sequencing was on average 0.4 mutation/molecule over the gap. One acid stable mutant, D7, was identified after the pH screening. Sequencing of this mutant revealed mutation H133Y originating from a mutation of the encoding triplet from CAC to TAC.

Mutant D7 was also found positive in the thermostability screening assay (Example 5).

DNA sequencing was performed on single stranded DNA

35 with a specific oligonuclectide designed to prime just before
the <u>SacII-ClaI</u> fragment. In a separate mutagenesis experiment

1000 Cm⁸ transformants were screened. Another acid stable

mutant, 2D5, was identified after the pH screening. This mutant has the following mutations:

H133Y CAC --> TAC

T149I ACA --> ATA

5 Bisulphite mutagenesis has been applied in a similar manner as just described on the <u>ClaI-Sal</u>I gap which runs from position 4569 to position 4976 of Figure 3. About 300 Cm⁵ transformants were screened (mutation frequency 0.6 mutations/molecule). No acid stable transformants were found.

10 A number of acid labile mutants were found. Among these acid labile mutants some may have a shifted pH spectrum resulting in a more alkaline stable phenotype.

Example 7

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Enzymatic mutagenesis of pMaTLia6

Single stranded pMaTLia6 (Pigure 4) was annealed with ClaI-SalI digested pMcTLia6 in order to obtain a heteroduplex running from position 4569 to 4976 (Figure 3). The gapped duplex was subjected to enzymatic misincorporation mutagenesis as described in the experimental section.

A sample obtained after dATP-limited primer elongation was split in three parts and incubated in the presence of reverse transcriptase with dCTP, dGTP and dTTP, respectively. After incubation at 37°C for 10 minutes a chase with all four dNTP's and Klenow polymerase was given T4-DNA ligase was added to finish the elongation to completely double stranded molecules.

These molecules were transformed into E. coli WK 6 Mut S and plasmid pools were recovered. These plasmid pools were subsequently transformed into E. coli WK 6 and the colonies were selected on chloramphenical (50 μ g/ml) containing agar plates. Resulting mutants were screened for stability of α -35 amylase as described in Example 5.

In another experiment the $\underline{Spel}-\underline{Sac}II$ gap was subjected to limited primer elongation with dATP, dCTP, dGTP and dTTP,

respectively. These primer pools were mutagenized by misincorporation (see experimental). 100 Cm transformants were tested on pH plates (Example 5) and mutant M29 was identified as more stable at low pH. The sequence of the 5 mutation was determined: AllIT GCG --> TCG

Example 8

<u>Properties of stable mutants</u>

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Table 1.

Two of the mutants obtained from the bisulphite mutagenesis experiments were further characterized. As described before DNA sequencing suggested the following amino acid replacements;

- 15 D7 contained a tyrosine at position 133 instead of a histidine (D7 = H133Y).
 - 2D5 contained the D7 mutation and in addition threonine 149 was replaced by isoleucine (2D5 = H133Y, T149I).

20 a) Measurement of enzymatic activity

The enzymatic activity of <u>B. licheniformis</u> α -amylase WT and mutants was measured using 4-nitrophenyl-maltopentaoside (4NP-DP5) as a substrate, 4 nitrophenol and maltopentaose are formed, this reaction can be followed by measuring the change in OD 405. The assay was performed at 35°C in 50mM MOPS, 50mM NaCl, 2mM CaCl₂ (PH 7.15) and 0-lmM 4NP-DP5.

Initial rates were measured and E-nitrophenol was taken as 10,000 l/M/cm. Figure 9 shows the results for WT and 2D5 α -

	Vmax	(µmol/min/mg)	Km (mM)	
	WI	66.7 ± 0.9	0.112 ± 0.005	
35	2D5	66.3 ± 0.7	0.119 ± 0.004	

30 amylases. Vmax and Km were calculated and are given in

Table 1

Table 1 clearly shows that the mutations of o-amylase 2D5 do not influence the enzymatic activity in a substantial way.

5 b) Influence of Ca2+ on the thermoinactivation

Heat inactivation experiments were performed for WT, D7 and 2D5 at varying calcium concentrations. The procedure was as follows:

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1) Demetallization

Enzyme (2 - 3 mg/ml) dialyzed for 24 hrs against

3 x 1 L 20 mM MOPS

5 mm EDTA

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5 mM EGTA pH 7.0

3 x 1 L ZOMM MOPS pH 7.0

2) Remetallization

20 - 500 μ l buffer 100 mM (e.g. MES, MOPS, EPPS)*

- 145 µl demetallized enzyme (e.g. 2.15 mg/ml)

- 100 µl CaCl₂ (100, 50, 30, 20, 10, 5 or 2.5 mM)

 $- \times \mu 1 K_2 SO_4 (100 mM)$

- (255-x) μ l H₂O

25

	[CaCl ₂] final	(mM)
)	0,25	14,75
	0,5	14,5
	1	14
	2	13
	3	12
5	\$	10
	10	O

- * pH MES e.g. 6.77 at room temperature will give 6.0 at 90°C (pKa 6.15 pKa/°C = -0.011)
 - pKa were from Table of Merck
 (Zwitterionische Puffersubstanzen)

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3) Heat-inactivation

- 1 ml enzyme solution preincubated at room temperature

 10 was heated at 90.5°C or 95°C in closed Pierce-vials
 (teflon coated-seals) at a concentration of about 0.2
 mg/ml 50 μl samples were withdrawn at regular intervals
 between 0 and 6 hrs with a syringe and cooled on ice.
 Residual activities have been determined with 4NP-DP5

 15 (0.5mM).
 - Half lives were determined using a single exponential decay fitting program (GRAPHPAD).
- Figures 10 and 11 show the half life times of WT and D7 a-amylases at pH 5.5 and 7.0 respectively as a function of the Ca²⁺ concentration at 90.5°C. The Ca²⁺ dependence of 2D5 has only been determined at pH 7.0 at 95°C (Figure 12). It can also be seen that the Ca dependence of the mutants is not different from that of the WT.

C. Thermostability of mutant α-amylases at different pH values

The pH dependence of thermoinactivation for both D7 and 2D5 has been determined at 90.5 and 95°C respectively using the buffer as described above at a 1 mM Ca²⁺ concentration. It can be concluded that the thermal stability of both D7 and 2D5 is greatly increased (up to twofold for 2D5) over the entire pH range. (Figures 13 and 14).

Example 9

Production of mutant enzymes in Bacillus

- Mutations in the B. <u>licheniformis</u> a-amylase, which were identified by expression in B. <u>coli</u> WK6 were transferred to a Bacillus expression vector in two different ways.
- **a**) With the aid of the unique restriction sites within the o-amylase gene (Figure 4), fragments carrying 10 mutations were isolated from pMaTLia6 mutants and subcloned into the homologous position of pBMa6.Lia6. The latter plasmid, which can be replicated either in E. coli or in Bacillus, was subsequently digested with SacI and recircularized with T4 DNA ligase. After 15 transformation into Bacillus subtilis 1A40 high level a-amylase production under control of the SPO, promoter was obtained. Recircularized pBMa6.Lia6 is named pB6.Lia6 to indicate the removal of the E. coli 20 portion of the vector.
- b) pBMa6.Lia6 single stranded DNA was recollected from E.

 COLI and annealed with restriction enzyme digested
 pBMc6.Lia6 double stranded DNA in order to obtain a
 gapped duplex with the intended gap on the a-amylase
 gene. This gap was then subjected to site-directed
 mutagenesis with an oligonuclectide (as described in
 the experimental section) which encodes the desired
 mutation. pBMc6.Lia6 vector is then transformed into
 pB6.Lia6 type vector as described above. Combination
 of different single site mutation can be performed by
 method a) if mutations are in different gaps,
 preferably, however, method b) is used.
- The mutations of mutants D7 and 2D5 were transferred to pBMa6.Lia6 by method a) by exchanging the <u>SacII-SalI</u> fragments and a-amylase was recovered from the medium of

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transformed <u>Bacillus subtilis</u> 1A40. Supernatants of both mutants were subjected to the screening procedures of Examples and it was confirmed that both mutants produce amylase which is more acid stable and more thermostable than 5 a-amylase produced by wild-type pB6.Lia6.

The phenotype of the a-amylase mutations in <u>Bacillus</u> is thus not different from the phenotype in <u>E. coli</u>.

Ultimately pB6.Lia6 mutants have been transformed into Bacillus licheniformis T9, which is a protease negative, a
amylase negative derivative of Bacillus licheniformis T5, (EP-0253455, CBS 470.83). Host T9 has been used to produce high level amounts of a-amylase mutants in a homologous system. The removal of the chromosomal a-amylase gene renders this strain very suited for the production of mutant a
amylase as no contaminating wild-type a-amylase is being produced anymore. Enzyme recovered from this strain has been used for industrial application testing. The industrial use of mutants pB6.Lia6.2D5 and pB6.Lia6.D7 was demonstrated.

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Example 10

Application test of mutant a-amylase under conditions of starch liquefaction

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To test mutant α -amylase 2D5 in more realistic circumstances, we have purified the fermentation broth (of Example 9) with ultrafiltration and formulated the enzyme with 50% propyleenglycol.

30 Three samples have been tested:

893701 : WT <u>B.licheniformis</u> T5 α -amylase 1530 TAU/g 893703 : 2D5 Mutant prepared as WT 2820 TAU/g Maxamyl 0819 Commercial sample 7090 TAU/g

35 One TAU (thermostable a-amylase unit) is defined as the quantity of enzyme that will convert under standardized conditions 1 mg of starch per minute in a product having an

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equal absorption to a reference colour at 620 nm after reaction with iodine. Standard conditions are pH 6.6; 30° C; reaction time : 20 min. Reference colour is 25g CoCl₂. 6H₂O, 3.84 g K₂Cr₂O, and 1 ml HCl (1M) in 100 ml destilled H₂O.

5

Liquefaction test at low pH (5.5 and 5.25)

The temperature of starch slurry is increased to 110 ± 10 0.5°C as quick as possible and kept at this temperature for 6 minutes.

The liquefaction is realized in continuous flow (5.4 l/h). 3 Samples of 135 ml (1.5 minute of liquefaction) are taken after 45, 60 and 75 minutes of liquefaction and kept at 95°C for two hours. After this time, 50 ml of the sample are acidified with 0.4 ml H₂SO₄ N to obtain pH 3.5 and put in boiling bath for 10 minutes in order to stop enzymatic activity before D.E. determination.

The remaining part of the sample is cooled in order to 20 determine residual enzymatic activity.
Slurry composition:

3.3 kg corn starch D.S. 88% (2.904 kg dry starch). 5.45 l well water (40 T.H.).

Dry substance of the slurry is 33%.

25 pH is corrected at 5.5 with 1N sulfuric acid or 1N NaOH.

Enzyme concentration: 4.4 TAU/gr dry starch.

The flow rate is verified two or three times during the trial.

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Determination of D.E.

Dry substance of liquefied starch is verified with a 35 refractometer (about 34%). D.E. is determined with the well-known Lane Eynon method. The results are shown in Figure 15.

3. Residual Enzymatic Activity

Residual amylase activity in liquefied starch is determined with a Brabender amylograph.

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40 g potato starch

390 ml distilled water at 50°C

50 ml Tris buffer 0.05 M pH 6.50

5 ml CaCl₂ 2H₂O at 30 g/l

10

The temperature is increased to 80°C (1.5°/min) when viscosity is stabilized (10 min) 5 ml of diluted liquefied starch (7 g up to 50 ml with distilled water) is added, the decrease of viscosity after 20 minutes is measured, this decrease is a function of the enzymatic activity. A standard curve with known enzymatic concentration allows to estimate residual activity in T.A.U.

Mutant 2D5 performs significantly better at pH < 5.5 and 110°C than WT enzyme. An improvement of 2-3 DE units at pH 5.25 is obtained with mutant 2D5.

Example 11

25 <u>Application test of mutant α-amylase under conditions</u> of textile desizing

To test the industrial application of alkaline aamylase mutants a test is performed on the stability at 20°C 30 in the following solution:

	1.4%	H ₂ O ₂ (35%)
	1.0-1.5%	Caustic Soda (100%)
	15-20 ml/l	Sodium Silicate (38 Bé)
3.5	0.3-0.5%	Alkylbenzene sulphonate (Lanaryl N.A
		ICI)
	0.5-1.0%	Organic stabilizer (Tinoclarite G)

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After incubation during 2.5 hours the a-amylase mutants selected for their desired properties should have any remaining enzyme activity.

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CLAIMS

- A mutant α-amylase, that is the expression product of a mutated DNA sequence encoding an α-amylase,
 Characterized in that the mutant α-amylase has an amino acid sequence which differs at least in one amino acid from the wild-type enzyme and that said mutant α-amylase exhibits improved properties for application in the degradation of starch and/or textile desizing wherein the improved
 properties are due to the amino acid replacements.
 - 2. An α -amylase according to Claim 1, characterized in that it exhibits improved thermostability.
- 3. An α-amylase according to Claim 1, characterized in that it exhibits improved stability at a pH below 6.5 and/or above 7.5.
- 4. An α -amylase according to Claim 1, characterized in 20 that it exhibits improved thermostability and acid stability.
- 5. An α-amylase according to any one of the Claims 1-4, in which the original gene from which the mutant enzyme is derived is obtained from a microorganism, preferably a 25 <u>Bacillus</u> strain.
- 6. An α-amylase according to Claim 5, in which said gene is derived from a wild-type gene of a strain selected from the group consisting of <u>B. stearothermophilus</u>, <u>B. 30 licheniformis</u> and <u>B. amyloliquefaciens</u>.
- 7. An α-amylase according to Claim 6, characterized in that this enzyme differs from the wild-type α-amylase obtainable from <u>Bacillus licheniformis</u> by an amino acid
 35 replacement at one or more of the positions 111, 133 and 149 or at corresponding positions in any homologous α-amylase.

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- 8. An a-amylase according to Claim 7, characterized in that it contains one or more of the following amino acid replacements: Ala-111-Thr, His-133-Tyr, Thr-149-Ile.
- 9. A mutant gene encoding an a-amylase as defined in any one of Claims 1-8.
 - 10. An expression vector which comprises a mutant gene according to Claim 9.
 - 11. A host cell harboring an expression vector according to Claim 10.
- 12. A host cell which is substantially incapable of l5 producing extracellular amylolytic enzymes prior to transformation, characterized in that it is transformed with an expression vector according to Claim 10.
- 13. A host cell according to Claim 12 being B.
 20 licheniformis T9.
- 14. A <u>Bacillus/E. Coli</u> shuttle vector, wherein the expression of the cloned gene in <u>E. Coli</u> is made impossible by physical separation of the regulatory sequences from the structural gene and wherein the expression of the cloned gene in <u>Bacillus</u> can be restored by digestion with a single restriction enzyme and subsequent recircularization.
- 15. A method for preparing an amylolytic enzyme having improved properties for application in starch degradation or in textile desizing which comprises the following steps:

mutagenizing a cloned gene encoding an amylolytic enzyme of interest or a fragment thereof;

isolating the obtained mutant amylase gene or genes;

introducing said mutant amylase gene or genes into a suitable host strain for expression and production;

recovering the produced mutant amylase and identifying those mutant amylases having improved properties for application in starch degradation or textile desizing.

- 5 16. A process for producing a mutant α-amylase comprising;
 - cultivating a host cell according to any of Claims 11-13 in a suitable medium.
 - recovering the produced a-amylase.

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- 17. Use of the α -amylase according to any one of the Claims 1-8 in starch degradation and in textile desizing.
- 18. Process for the degradation of starch which 15 comprises the use of a mutated α-amylase according to any one of the Claims 1-8.
- 19. Process for textile desizing which comprises the use of a mutated a-amylase according to any one of the Claims 20 1-8.
 - 20. Starch degradation composition comprising a mutated α-amylase according to any one of the Claims 1-8.
- 25 21. Textile desizing composition comprising a mutated α -amylase according to any one of the Claims 1-8.

10	20	30	40	50	60
AATTCACCTCGAA					
70 TTTTTTGGAGATT	80	90 	100	110	120
130 GAAAGCAAGCTGA	140 TAAACCGATAG	150 Caattaaagg	160 TCCTTTCC/	170 Geerrere	180 TTOGAG
190 ATTTTCAACGTGA	200 AAAAATTATT/	210 ATTCGCAATTO	220 CAAGCTCTG(230 Cregegeari	240 TCGGTG
ATGACGGTGAAAA	260 CCTCTGACAC	atgcagetec:	CGAGACGGT	CACAGCTTGT	TOTAAG
310	320	330	สผิด	350	360
CGGATGCAGATCA	CGCGCCCTGT	ngcgőőgcat.	TAAGCGCGGC	acréfécte	MTACGC
370	380	390	400	410	420
GCAGCGTGACCGC	TACACTTGCC	AGCGCCCTAG	CGCCCGCTCC	TTTCGCTTTC	TTCCCTT
430	440	450	460	470	480
CCTTTCTCGCCAC	GTTCGCCGGC	rrrccccarc.	AAGCTCTAAA:	rcoggggcrc	CCTTTAG
490	500	510	520	530	540
GGTTCCGATTTAG	HGCIIIACCO	UNCCICUNCO	UCAAAAAACT,	IGATTAGGUT	WIREII.
550 CACGTAGTGGGCC	560 Americantes	570	580	590	600
610 TCTTTAATAGTGG	620 ACTOTTOTTO	630 Caaactggaa	640 caacactcaa	650 CCTATOTOG	660 TICTATI
CTTTIGATTIATA	680 agggattito				
720	740	780	ማደሰ	270	ಌ೩೧
AACAAAATTTAA	CGCGAATTIT	aacaaaatat	TAACGITTAC	AATITGATCT!	acacrea
790	800	810	820	830	840
gregriegerge					
850	860	870	880	890	900
GAATCAGGGGATA	LACGCAGGAAA	GAACATGTGA	OCAAAAGGCC.	AGCAAAAGGC	CAGGAAC
910	920	930	940	950	960
CGTAAAAAGGCCC	CGTTGCTGGC	GPTTTTCCAT	AGGCTCCGCC	DECETGACGA	GCATCAC
	980				
AAAAATCGACGCT					
1030 TTTCCCCCTGGA/	1040 Gereceteat				
1090 CTGTCCGCCTTTC		1110 AAGCGTGGCG	1120 CTTTCTCAAT		1140 TAGGTAT

1150 CTCAGTTCGGTG	1160	1170	1180	1190	1200
	1220				
CCCGACCGCTGC	3CCTTATCCGC	itaactatogt	CTTGAGTCCA	ACCCGGTAAC	IACACGAC
1270 TTATCGCCACTG	1280 GCAGCAGCCAC	1290 TGGTAACAGG	1300 ATTAGCAGAG	1310 CGAGGTATOI	1320 'AGGCGGT
1330 GCTACAGAGTTC	1340	1350	1360	1370	1380
1390 ATCTGCGCTCTGK	1400 TGAAGCCAGI	1410 Taccttcgga	1420 AAAAGAGTTG	1430 GTAGCTCTTG	1440 MTCCGGC
1450	1460	1470	1480	1490	1500
AAACAAACCACC					
1510 AAAAAAGGATCTY	1520 Caagaagatco	1530 TITGATCTTT	1540 TCTACGGGGT	1550 CTGACGCTCA	1560 GTGGAAC
1570	1580	1590	1600	1610	1620
GAAAACTCACGT	raagggattti	'GOTCATGAGA	TTATCAAAAA	GGATCTTCAC	CTAGATO
1630 Ctittaaattaa/	1640 vaatgaagiti	1650 Taaatcaatc	1660 Taaagtatat	1670 ATGAGTAAAC	1680 TIGGICT
1690	1700	1710	1720	1730	1740
GACAGTTACCAAT	TOCTTAATCAG	TGAGGCACCT	ATCTCAGCGA	TCTGTCTATT	TCCTTCA
1750 TCCATAGTTGCC	1760 rgactoccom	1770 CCTCTAGATA	1780 ACTACGATAC	. 1790 GGGAGGGCTT	1800 ACCATOT
	1820				
GGCCCCAGTGCTC	CAATGATACC	GCGAGACCCA	CCCTCACCGG	CTCCAGATTI	'ATCAGCA
1870 ATAAACCAGCCAC	1880	1890	1900	1910	1920
1930 ATCCAGTCTATT/	1940 VATTGTTGCCG	1950 GGAAGCTAGA	1960 Gtaagtagtt	1970 CGCCAGTTAA	1980 TAGTTIG
1990	2000	2010	2020	2030	2040
CGCAACGTTGTT(CCATTGCTGC	AGGCATCGTG	GTGTCACGCT	COTCOTTTOC	TATOOCT
2050 TCATTCAGCTCC	2060 SGTTCCCAACG	2070 ATCAAGGCGA	2080 GTTACATGAT	2090 CCCCCATGIT	2100 GTGCAAA
2110	2120	2130	2140	2150	2160
AAAGCGGTTAGCT	rccttccctcc	TCCGATCGTT	GTCAGAAGTA	AGTTOGCCOC	ACTOTTA
2170 TCACTCATGGTT/			2200 CTTACTGTCA		
2230	2240	2250	2260	2270	2280

Fig. 1 (continued)

2390 2300 2310 2320 2330 2340 2360 2370 2380 2390 2400 2350 2350 2360 2370 2380 2390 2400 2450 2550	TITTCTGTGACTG	GTGAGTACTC/	\ACCAAGTCAT	TCTGAGAAT/	\GTGTATGCG(CCACCC
2350 2360 2370 2380 2390 2400 GTGGTCATCATTGGAAAACGTTOTTCGGGGGGAAAACTGTCAAGGATCTTACCGCTGTTG 2410 2420 2430 2440 2450 2460 AGATCCAGTTCGATGAACCCACTCGTGCACCCAACTGATCTTTACTTTC 2470 2480 2490 2500 2510 2520 ACCAGCGTTTCTCGGTGAGCAAAAACGGAAGGCAAAAATGCCGCAAAAAAGGGAATAAGG 2530 2540 2550 2560 2570 2580 GCGACACGGAAAATGTGAATACTCATACTCTTCTTTTTTCAATATTATTGAACCAGACAGCAG 2590 2600 2610 2620 2630 2640 TTTTATTGTTCATGATAATAATTATTTTTTTTTTTTTTT	2290	2300	2310 Schoolataata	2320 (CCGCGCCAC)	2330 ATAGCAGAAC	
CTGCTCATCATTGGAAAACGTTCTTCGGGGGCGAAAACTCTCAAGGATCTTTACCCCTGTTG 2410 2420 2430 2440 2450 2460 AGATCCAGTTCGATGTAACGCAACTCGTGCACCCAACTGATCTTCAGCATCTTTACTTTC 2470 2480 2490 2500 2510 2520 ACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAAGGGAATAAGG 2530 2540 2550 2560 2570 2580 CCGCACACGGAAATGTTGAATACTCTATACTCTTCCTTTTTCAATATTATTTGAAGCAGGACAG 2590 2600 2610 2620 2630 2640 TTTTATTCTTCAGATGATATATTTTTTACTTGTGCAATGTAACATCAGAGGATTTTGAGA 2650 2660 2670 2680 2690 2700 CACAAACGTGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGAGCTCCCCCGCGCGCG	2350	2360	2370	2380	2390	2400
AGATECAGTTEGATGTAAGCCACTCGTGCACCCAACTGATGTTCAGCATCTTTACTTTC 2470 2480 2490 2500 2510 2520 ACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGCCAAAATGCCGCAAAAAAAGGGAATAAGG 2530 2540 2550 2560 2570 2580 CCGCACACGGAAATGTTGAATACTCTATACTCTTCTTTTTCAATATTATTGAAGCAGGACAGG 2590 2600 2610 2620 2630 2640 TTTATTGTTCATGATGATATATTTTTACTTGTGCAATGTAACATCAGAGATTTTGACA 2650 2660 2670 2680 2690 2700 CAGAACGTGGCTTGTTGAAAAAAAAGCCCCGCTCATTAGGCGGGCTAAAAAAAA	GIGCTCĂĬĊĂTTC	gaaaācgtto.	rtoggggcga/	laacīctcaa:	GGATCTTACC	CTGTTG
2470 2480 2490 2500 2510 2520 ACCAGCGITTCTGGGTGAGCAAAAACAGGAAGGCAAAAATGCCGCAAAAAAAGGGAATAAGG	2410 AGATOCAGTTOGA	2420 TOTAACCCAC	2430 regreeacce	2440 Vactgatett	2450 CAGCATOTTT	2460 TACTTIC
ACCAGOGITTCTGGGTGAGCAAAAAAGGGAAGGCAAAAATGCCGCAAAAAAAGGGAATAAGG 2530 2540 2550 2560 2570 2580 CCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCAGACAG 2590 2600 2610 2620 2630 2640 TTTTATTGTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGGATTTTGAGA 2650 2660 2670 2680 2690 2700 CACAACGTGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGACTCCCCGCGCGCG						
COCIACACGGAAATGTTGAATACTCATACTCTTCCTTTTTTCAATATTATTGAACACAGAGA 2590 2600 2610 2620 2630 2640 TTTTATTGTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGA 2650 2660 2670 2680 2690 2700 CACAACGTGGCTTTGTTGAATAAATCGAACTTTGCTGAGTTGACTCCCCGCGCGCG	ACCAGCGITICIC	240U IGGTGAGCAAA	aacaggaaggi	CAAAATGCCG	CAAAAAAGGG	AATÄÄGG
2590 2600 2610 2620 2630 2640	2530	2540	2550	2560	2570	2580
2650						
2650 2660 2670 2680 2690 2700 CACAACGTGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGACTCCCCGCGCGCG	2590	2600	2610	2620 roca arora a	2630 catcagagat	2640 TTTGAGA
CACAACGTGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGACTCCCCGGGGCGATGA 2710 2720 2730 2740 2750 2760 GGTCGAATTTGCTTTCGAAAAAAAAGCCCGCTCATTAGGCGGGCTAAAAAAAA						
2710 2720 2730 2740 2750 2760 GGTCGAATTTGCTTTCGAAAAAAAAGCCCGCTCATTAGGCGGGCTAAAAAAAA	2650 Cacaacctgcct	2660 Pegetgaataa	2670 atcgaactit	2680 TGCTGAGTTG	2690 ACTCCCCGGG	Z700 CGCGATG
### CONTINUATION C						
2830 2840 2850 2860 2870 2880 AACCAGGCGTTTAACGCCACCAATAACTGCCTTAAAAAAAA	2710 GGTCGAATTIGC	2720 ITTCGAAAAA	AAGCCCGCTC	ATTAGGCGGG	CTAAAAAAAA	GCCCGCT
2830 2840 2850 2860 2870 2880 AACCAGGCGTTTAAGGGCACCAATAACTGCCTTAAAAAAATTACGCCCGGCCCTGCCACT 2890 2900 2910 2920 2930 2940 CATCGCAGTACTGTTGTAATTCATTAAGCCATTCTGCCGACATGGAAGCCATCACAGACGG 2950 2960 2970 2980 2990 3000 CATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATTTGC 3010 3020 3030 3040 3050 3060 CCATAGTGAAAACGGGGGGGAAGAAGATTGTCCCATATTCGCCACCTTTAAATCAAAAACTGG 3070 3080 3090 3100 3110 3120 TGAAACTCACCCAGGGATTGGCTGAGACGAAAAAACATATTCTCAATAAACCCTTTAGGGA 3130 3140 3150 3160 3170 3180 AATAGGCCAGGTTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAAACGTTTCAGTTTGCTCATGGAAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATAC	2770	2780	2790	2800	2810	2820
2890 2900 2910 2920 2930 2940 CATCGCAGTACTGTTGTAAATCAGTAAGCATTCTGCCGACATGGAAGCCATCACAGACGG 2950 2960 2970 2980 2990 3000 CATGATGAACCTGAATCGCCAGCGGCATCACACACACCTTGTCGCCTTGCGGTATAATATTTGC 3010 3020 3030 3040 3050 3060 CCATAGTGAAAACGGGGGGGAAGAAGTTGTCCCATATTCGCCACGTTTAAATCAAAAACTGG 3070 3080 3090 3100 3110 3120 TGAAACTCACCCAGGGGATTGAGACGAAAAACATATTCTCAATAAACCCTTTAGGGA 3130 3140 3150 3160 3170 3180 AATAGGCCAGGTTTTCACCGTAACACCCCCACATCTTGCGAATATTTGTTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAAACGTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACCTCATTCCCATATCCCATTTGCCATTGCCATACACACCCCTCATTGCAAAAACTGCC	CATTAGGCGGGC	TCGAATTTCTC	CCATTCATCC	CCTTATTATC	ACTTATTCAC	GCGTAGC
2890 2900 2910 2920 2930 2940 CATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACAGACGG 2950 2960 2970 2980 2990 3000 CATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATTTGC 3010 3020 3030 3040 3050 3060 CCATAGTGAAAACGGGGGGGAAGAAGTTGTCCCATATTCGCCACGTTTAAATCAAAAACTGG 3070 3080 3090 3100 3110 3120 TGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAAACCTTTAGGGA 3130 3140 3150 3160 3170 3180 AATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGGCGATGAAAAACGTTTCACTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACCTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATAC	2830 AACCAGGGGTTT	2840 aagggcaccaa	2850 Taactgcott	2860 Aaaaaaatta	2870 .000000000	2880 TGCCACT
2950 2960 2970 2980 2990 3000 CATGATGAACCTGAATCGCCAGCGGCATCACCACGACGG 3010 3020 3030 3040 3050 3060 CCATAGTGAAAACGGGGGGGAAGAAGATTGTCCATATTTGCCCATAGTGAAAACGGGGGGGG						
3010 3020 3030 3040 3050 3060 CCATACTGAAAACGGGGGGGGGGGGGGGGGGAGAAATTGGCCACGTTTAAATCAAAACTGG 3070 3080 3090 3100 3110 3120 TGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCGTTTAGGGA 3130 3140 3150 3160 3170 3180 AATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACCTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGACGTGAACACTATCCCATATCACCAGCTGACCGTCTTTCATTTGCCATAC	2890 CATCGCAGTACT	ZYUU GITGTAATTCA	TTAAGCATIC	TGCCGACATC	GAAGCCATC/	CAGACGG
3010 3020 3030 3040 3050 3060 CCATACTGAAAACGGGGGGGGGGGGGGGGGGAGAAATTGGCCACGTTTAAATCAAAACTGG 3070 3080 3090 3100 3110 3120 TGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCGTTTAGGGA 3130 3140 3150 3160 3170 3180 AATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACCTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGACGTGAACACTATCCCATATCACCAGCTGACCGTCTTTCATTTGCCATAC	2950	2960	2970	2980	2990	3000
3070 3080 3090 3100 3110 3120 TGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCCTTTAGGGA 3130 3140 3150 3160 3170 3180 AATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACGTTCCATTGCCATAC						
3070 3080 3090 3100 3110 3120 TGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCCTTTAGGGA 3130 3140 3150 3160 3170 3180 AATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACGTTCCATTGCCATAC	3010	3020	3030	3040	3050	3060
TGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCCTTTAGGGA 3130 3140 3150 3160 3170 3180 AATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACCTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATAC	CCATAGTGAAAA	CGGGGGCGAAG	iaagitgivu	MATTOGCOAL	WIIIIWWII.	unno i co
3130 3140 3150 3160 3170 3180 AATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACTATCCCATATCACCAGGTCACCGTCTTTCATTGCCATAC	3070	3080	3090	3100	3110	
AATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC 3190 3200 3210 3220 3230 3240 GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATAC	TGAAACTCACCC					
GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACCTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATAC	3130 AATAGGCCAGGT	3140 TTTCACCGTA	3150 ACACGCCACAS	3160 CTTGCGAAT/	3170 VTATOTOTAC	3180 NAACTGCC
GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAA 3250 3260 3270 3280 3290 3300 CGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATAC	aton	รว กก	3210	3220	7270	3240
COCTOTAACAACCCTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATAC	GGAAATŐĞŤŐGI	GGTAŤŤČĀCT	CCAGĂGCGATI	JAAAĀCGTTT	CAGTTTĞCTC.	
and the second s	3250	3260	3270	3280	3290	3300
2740	COGTOTAACAAC					
3310 3320 3330 3340 3350 3360 GAAATTCCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGGCCGGATAAAACT	3310 GAAATTCCGGAT	3320 rgagcattcat	3330 CAGGCGGGCA	3340 agaatgtgaa	3350 TAAAGGCCGG	3360 Ataaaact

skrindskrinskri	3370 TTATTTTICT	3380	3390 TAAAAAGGCC	3400 Traatatcca	3410 GCTAAACGGTY	3420 CTGGT
7(37(20)		3440				
TATAG	GTACATTGAG	CAACTGACTG	aaatocctca	aaatottott	TACGATGCCA	rrggg
\$ 999 \$ 999 8 3	3490 TCAACGGTGG	3500	3510	3520 Togattitag	3530 CITCOTTAGO	3540 rccra
wiwiw	TOWNCOOLOG	TWTWTCOWN.		7 mm117 4 7 21 mm		* ****
	3550	3560	3570	3580	3590	3600
AAAAT	ctccataact	CAAAAAATAC	gcccggragr	GATUTTATTT	Cattaiggig	WWW
	3610	3620	3630	3640	3650	3660
TGGAA	CCTCTTACGT	GCCGATCAAC	GTETCATTI	CGCCAAAAGI	TGGCCCAGGG	CLICU
	3670	3680	3690	3700	3710	3720
CGGTA	TČAACAGGGA	CACCAGGATT	Tattiatici	GCGAAG1GA1	CITCORTORC	MMATH
	3730	3740	3750	3760	3770	3780
TTTAT	TCGAAGACGA	AAGGGCATCG	CGCGCGGGGA	ATTCCCGGGG	IATCCGTCGAC	UTUCA
GCCAA	3790 GCTTGGTCTA					

Fig. 1 (continued)

GTCTACA	10 IAACCC	CITA	20 Aaaac	arri	30 TTAAA	i .agc	TTT:	40 Taag	ccarc	50 TOTA) C art e	CTI	60 'AAG
					90								120
GAATTCA	CACTG	gcci	TGGTT	AAGG	TTAAC	ATG	TGG	ACGG	AATGG	CTAA	AGTGI	'AGT	'AAA
GTACAAT	130 TAATC	GGGA	140 GCTTA	GATG	150 TCCCT) TCA	ACA:	160 ICTI	atata	170 GAAG	O GGAAC		180 GGC
AAATGGA	190 WATTO	AAAC	200 Iaatta	ACGA	210 GCATA) .CAG	Taa,	220 AATT	TTATA	230 Tata	O TTACC		240 ATA
	250		260		270	\		280		290	2		300
TTGAAGA	TCGCG	GITI	TGACA	GAGA	AGAAA	TII	GGT	ATAA	ccara	AGCG	CAGTO	iaac	AAC
TTTTCTC	310 GGAAG	TCAT	320 YGGATG	aacti	330 TCATO) IAAG	AAG.	340 AGGA	ATTCO	350 AGCTI) 	caa	360 GAT
	370		380		390	}		400		410	3		420
CCAAGGA	IGGTGA	TCTA	GAGTC	atga	AACAA K Q	CAA	AAA	CGGC	TTTAC	acce:	CATTO	cra	ACC
													Y
CTGTTAT	430 ***********	و مارشون	440	ساتساملها	450	; ; ;	انتاستانان	460	n simm	174 17000) ^*****	دارمانداندرار	480
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	490		500		810	}		%20		*) ¤20			ನಟಣ
GGGACGC	TGATG	Cagi	ATTTT	gaat	GGTAC	ATC	ccc.	aatg	ACGGC	CAAC	ATTGC	AAG	ccr
G T 5	L M	Q	YF	E	M A	M	P	N	D G	Q :	3 W	K	R
99999 A A A	550		560		570)		580		590)		600
TTGCAA/ L Q													
25													
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77	670		680		690) .		700		71	٥		720
GGGGAGT	TTCAT	Caai	laaggg	accc	mrcac	IACA	aag	TACG	GCACA	aaag:	GAGAC	CTG	
G E 65	F H	**	<i>E</i> 0	1	v n	3	Ω	X	u I	<i>1</i> /4	Ca SS	مذ	Ų
mmmmm	730	x immed	740		750)		760					780
TOTGOGA S A													
85													
CACAAA	790 300000	GCT			810 CAAGA								840 GAC
H K													
105	850		860		870)		880) .	89	0		900
CGCAAC	CGCGTA	ATT.	rcagga	GAAC	ACCTA	LTA	AAA	GCCT	'GGACA	CATT	TTCAT	TI	
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Fig. 2 (continued)

10 AATTCACCTCGAA	20 AAGCAAGCTGA	30	40 10447744	50	60
70 TTTTTTCGAGAT					
130 GAAAGCAAGCTO/	140 VTAAACCGATA	150 Caattaaage	160 ETCCTTTIGG	170 AGCCTTTTT	180 TTTGGAG
	200	210	220	230	240
250	260	270	280	290	300
ATGACGGTGAAAA	CCTCTGACAC	ATGCAGCTCC	CGGAGACGGT	CACAGCTTOT	CTGTÃAG
310 CGGATGCAGATCA	320 NOCCECCTOT	330 AGCGGCGCAT	340 TAAGCGCGGG	350 GGGTGTGGTG	360 GITACGC
370	380	390	400	410	420
GCAGCGTGACCGC					
CCTTTCTCGCCAC	440 XITTCGCCGGC	450 TTTCCCCGTC	460 Aagctctaaa	470 ICCGGGGGTC	480 CCTITAG
490 GGTTCCGATTTAC	500 MGCTTTACGG	510 CACCTCGACC	520 CCAAAAAACT	530 Igattagggt	540 BATOGIT
	560	570	580	590	600
	620				
TOTTTAATAGTGG	ACICTTOTTO	CAAACTGGAA	Caacactcaai	COTATOTOG	JICTATT
670 CTTTTGATTTATA	680 Jagggattitg	690 CCGATTTCGG	700	710	720
	740				
AACAAAATTTAA	CGCGAATTTT.	AACAAAATAT	TAACGTTTAC.	aattigatet(acacrca
790 GICGITCGGCIGC	800 GGCGAGCGGT	810 ATCAGCTCAC	820 TCAAAGGCGG	830 Faatacgget/	840 NTCCACA
850 GAATCAGGGGATA	860 LACGCAGGAAA	870 gaacateetga	880 GCAAAAGGCC	890	900
910	920	930	940	950	960
CGTAAAAAGGCCC					
970 AAAAATCGACGCT	980 Caagtcagag	990 GTGGCGAAAC	1000 CCGACAGGAC	1010 Pataaagata	1020 CCAGGCG
1030 TTTCCCCCTGGAA	1040 Gerecerean	1050 3000TCTCCT	1060	1070	1080
1090 CTGTCCGCCTTTC	1100 TCCCTTCGGG	1110 AAGCGTGGCG	1120 CITTCTCAAT	1130 Ictcacgctgi	1140 TAGGTAT

1150	1160	1170	1180	1190	1200
CTCAGTTCGGTG	raggregree	CTCCAAGCTC	GGCTGTGTGC	ACGAACCCCC	COTTCAG
1210	1220	1230	1240	1250	1260
CCCGACCGCTGC	CCTTATCCGC	MAACTATOGI	CITGAGICCA	ACCCGGTAAC	ACACGAC
1270	1280	1290	1300	1310	1320
TTATCGCCACTGC					'AGGCĞGT
1330	1340	1350	1360	1370	1380
GCTACAGĂĞTTC	TGAAĞTGGTC	GCCTĂĂCTAC	GGCTACACTA	gaaggacagt	ATTTÖGT
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1630 CTTTTAAATTAA	1640 Vaatgaactii	1650 Taaatcaatc	1660 Taaagtatat	1570 ATGAGTAAAC	1680 Tragmer
1690 GACAGTTACCAAT	1700 roomaandad	1710 maggrager	1720 'aromoacoca	1730 TOTOTATI	1740
1750 TCCATAGTTGCCT	1760	1770	1780	1790	1800
1810	1820	1830	1840	1850	1860
GOCCCCAGTGCT					
1870	1880	1890	1900	1910	1920
ATAAACCAGCCAG					cecerce
1930		1950	1960	1970	1980
ATCCAGTCTATT	attgrigeei	IGGAAGCTAGA	igiaagtagti	CGCCAGITAA	TAGITTO
1990		2010	2020	2030	2040
CGCAACGTTGTT	CCATTGCTGC	CAGGCATCGTC	GTOTCACGCI	CGICGIITGC	MATOGCT
			2080		
TCATTCAGGTCC	COTTCCCAAC	IATCAAGGCGA	GTTACATGAT	CCCCCATGIT	TOTGCAAA
2110	2120	2130		2150	
AAAGCGGTTAGC	CCTTCGGTCC	TCCGATCGT	GTCAGAAGTA	AGTTGGCCGC	AGTOTTA
2170	2180	2190	2200	2210	2220
TCACTCATGGTT					
2230	2240	2250	2260	2270	2280
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Fig. 3 (continued)

TITTCTGTGACTC	KTGAGTACTC	Caaccaactc/	ittctgagaat	'AGTGTATGC	CCCACCG
2290	2300	2310	2320	2330	2340
AGTTGCTCTTGCC	CGGCGTCAAC	ACGGGATAAT	'ACCGCGCCAC	ATAGCAGAA	TITTAAAA
2350 GTGCTCATCATTO	2360	2370	2380	2390	2400
GIGCICATCATTO	GAAAACGTTC	TTCGGGGCGA	AAACTCTCAA	GGATOTTACC	CCTGTTG
2410	2420	2430	2440	2450	2460
AGATCCAGTTCGA					
2470	2480	2490	2500	2510	2520
weenstranger in the	uulumulmma	AACAGGAAGG	Caaaatuccu	CAAAAAAGGC	iaataagg
2530	2540	2550	2560	2570	2580
GCGACACGGAAAT					
2590	2600	2610	2620	2630	2640
TITTATIGITCAT					
2650 CACAACGTGGCTT	2660 TOTTOS STA S	2670 arceaacrim	2680	2690 1 <i>0</i> 00000	2700
Z710 GGTCGAATTTGCT	2720 TTC//AAAAAA	2730 AAGCCCCCTT	2740 arraggegee	2750 ~~~~~~	2760
2770 CATTAGGCGGGCT	2780 CGAATTTCTG	2790 CCATTCATCC	2800 OCTTATTATC	2810 Activativa	2820
AACCAGGCGTTTA	2040 AGGGCACCAA	2050 Taactgcctt	2860 Aaaaaaatta	2870 CGCCCCCCCC	2880 Toccact
CATCGCAGTACTG	zyuu Tigiaattca	zyłu Ttaagcattc	2920 TGCCGACATG	2930 GAAGCCATCA	Z940 CAGACGG
			2980		
CATGATGAACCTG	AATCGCCAGC	GGCATCAGCA	COLLCICCCC	2990 ITGCGTATAA	COUC TATTTCC
3010	รกวก	3030	จกมก	3050	2060
CCATAGTGÃÃAAC	GGGGGGAAG	aagitgicca	TATTCGCCAC	STITAAATCA	3060 aaactgg
3070	3080	OPOF	3100	2110	3120
TGAAACTCACCCA	GGGÄTTGGCT	GAGÃCGAAAA	acataticic.	atăaaccet	TTAĞĞĞA
3130	3140	3150	3160	3170	3180
AATAGGCCAGGTT	TTCACCGTAA	CACGCCACAT	CTTGCGAATA'	PATOTOTAGA	AACTGCC
3190	3200	3210	3220	3230	3240
GGAAATCGTCGTG	GTATTCACTC	CACAGCGATG	AAAACGTTTC/	ngittäctca	TOGAAAA
3250	3260	3270	3280	3290	3300
COUTOTAACAAGO	OTGAACACTA'	TCCCATATCA	CCACCTCACC	TCTTTCATT	GCCĂŤAC
3310 GAAATTCCGGATG	3320 AGCATTCATO	3330 AGGCOOOCA A	3340 Gaatotoaati	3350	3360

Fig. 3 (continued)

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		300	CGA'	TTG: L	MG	ACG(MG	27.8	Jelel	aca	CIC.	atc	mc	TTG	cro	CCI	CAT	TCI	CGCAG
		SCC A	CGA'	TTG(L	L L	ACG(T	r	TTA L	F	aca A	CTC. L	ATC I	rtc F	TTG L	ctg L	CCT P	CAT H	TCI S	rgcag A
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CAC A	Y BCG A	39 39 36 40 40	CGA' R 70 GCA A *1 30 CAT	TTCK L AAT N TCC	IIG/ L 35 III L 46 AAG	ACGG T 980 AATG N 040 CGT	TIG L G G	ITA L ACC T	399 399 CTG L 405	GCG A O ATC M O GAC	CTC. L CAG Q	ATC I 40 TAT Y 40 GCA	FTC F DO FTT F 60 TAT	TTG L GAA E	CTG L 4 TGG W 4 GCI	CCT P O10 TAC Y O70 GAA	CAT H ATC M	TCI S CCC P	IGCAG A 4020 JAATG N
CAC A ACC D	Y BCG A	39 39 36 40 40	CGA' R 70 GCA A *1 30 CAT	TTCK L AATK N	IIG/ L 35 III L 46 AAG	ACGG T 980 AATG N 040 CGT	TIG L G G	ITA L ACC T	399 399 CTG L 405	GCG A O ATC M O GAC	CTC. L CAG Q	ATC 40 TAT Y 40 GCA	FTC F DO FTT F 60 TAT	TTG L GAA E	CTG L 4 TGG W 4 GCI	CCT P O10 TAC Y O70 GAA	CAT H ATC M	TCI S CCC P	A 4020 SAATG N 4080
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Fig. 3 (continued)

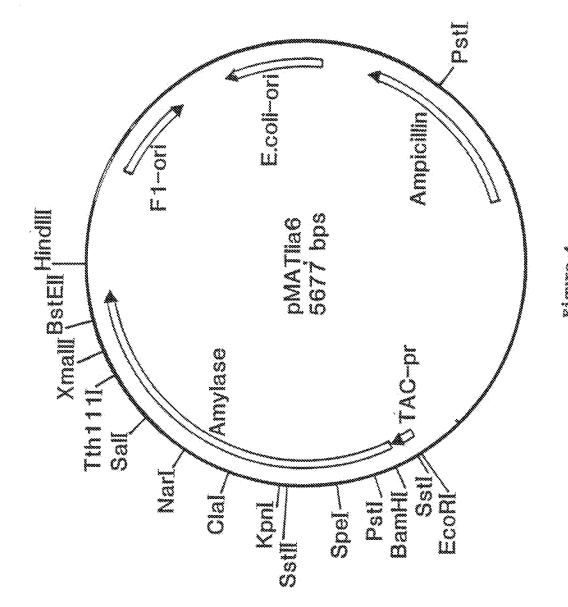


Figure 4

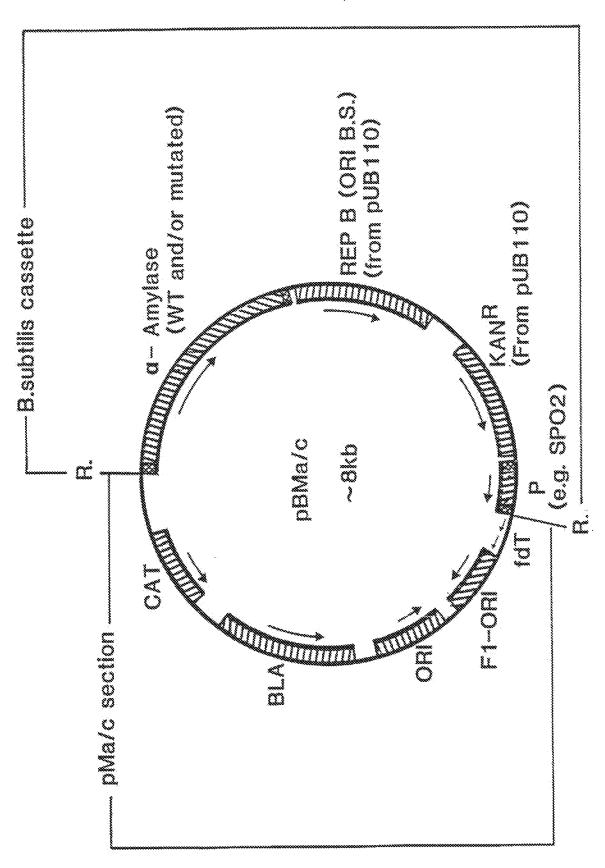


Figure 5

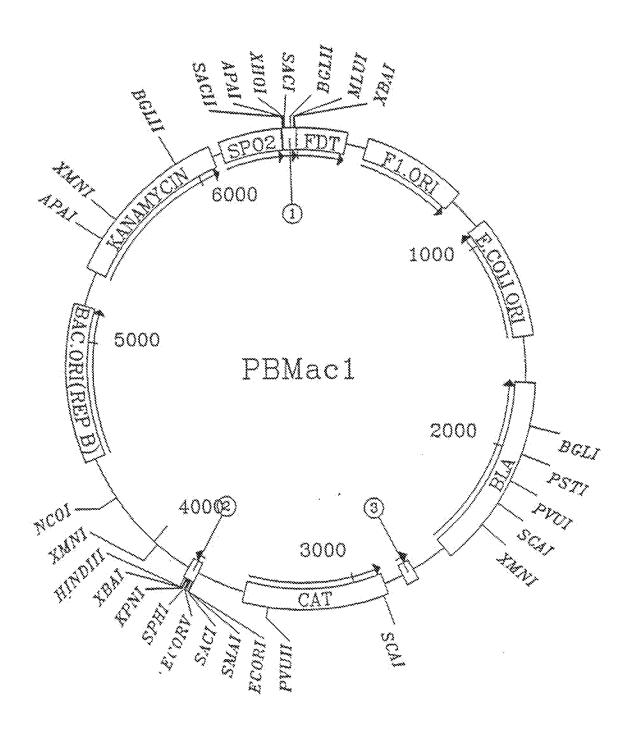


Figure 6

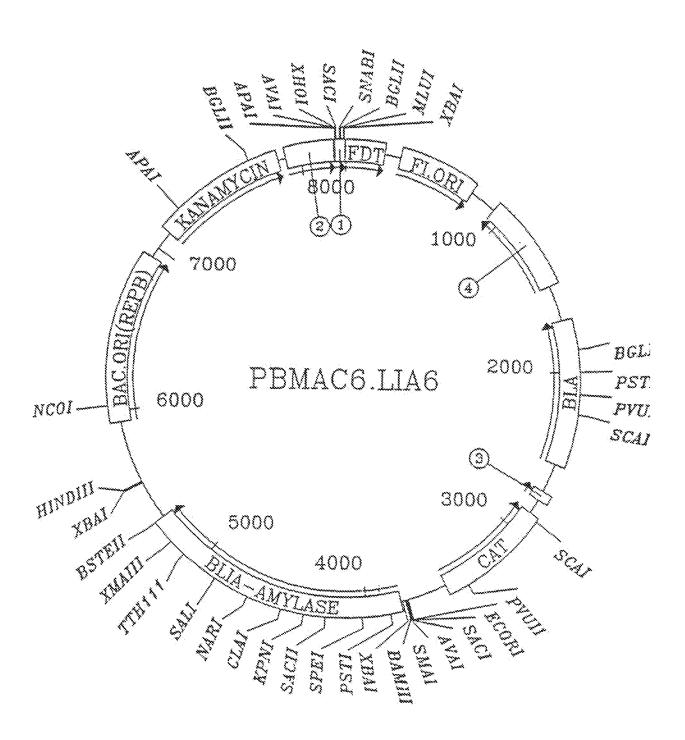


Figure 7

EcoRi

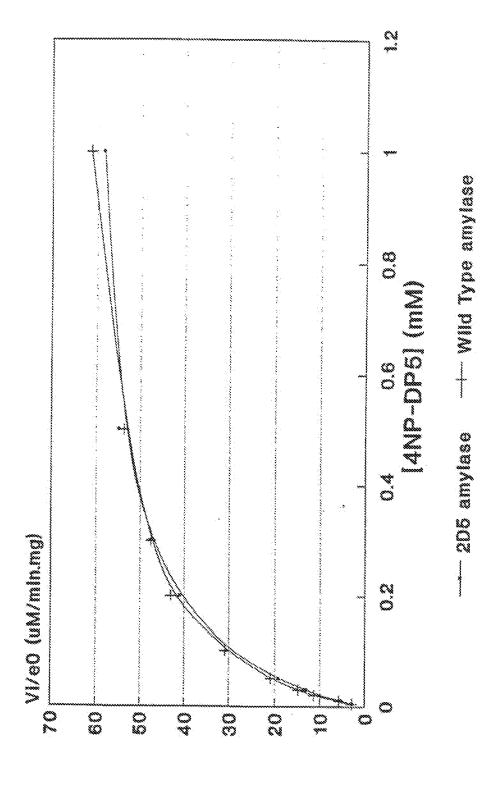
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BamHI

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CCTCTCACAAAAGCG GCAAAT
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Figure 8



Elgure 8

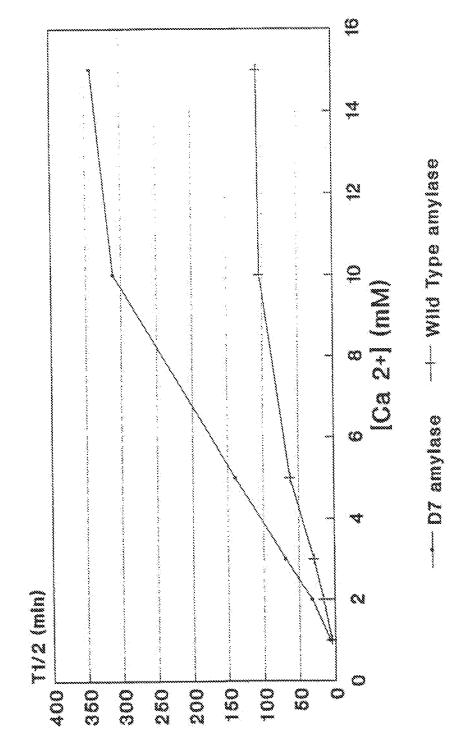
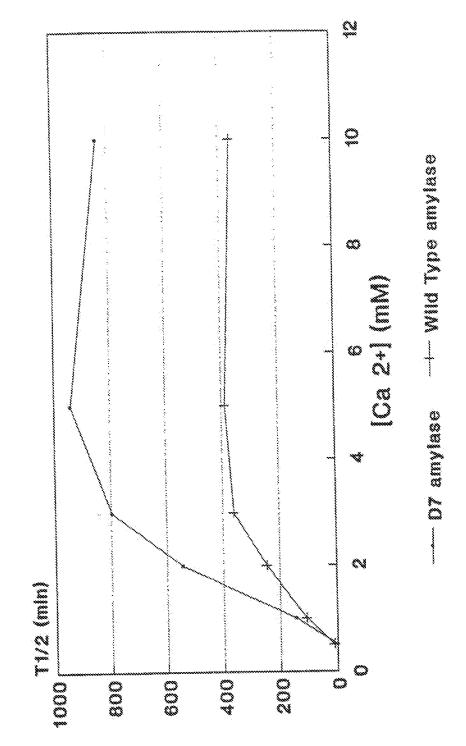


Figure 10



Eigure I

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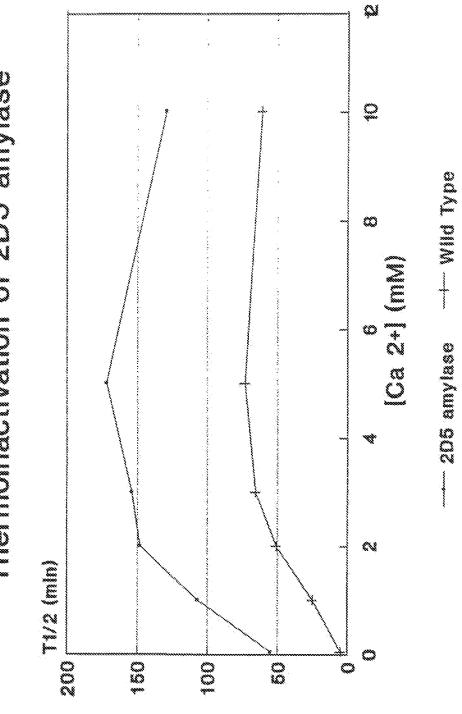
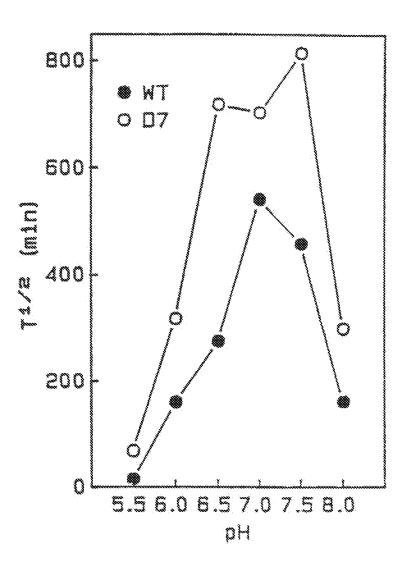


Figure 12

WO 91/00353



Pigure 13

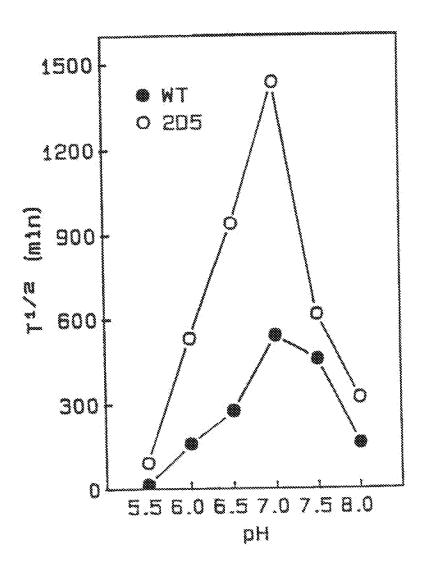
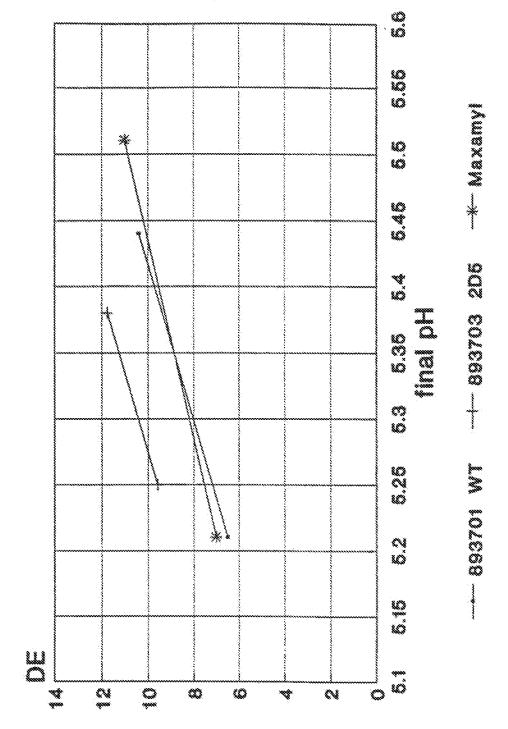


Figure 14



Figure